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14. ABSTRACT

CBP and p27KIP1 are two genes that are highly relevant to human prostate cancer. The objective of this proposal is to test the hypothesis that deletion of both alleles of CBP, in conjunction with deletion of one allele of p27KIP1, will result in the development of high-grade tumor in the prostate. The specific aims are to create CBP/p27KIP1 double knockout mice, to determine whether they present enhanced tumor progression compared to CBP single knockout mice alone, and to determine whether the application of two drugs, bortezomib and suberoylanilide hydroxamic acid (SAHA), are effective for the treatment of prostate tumors developed in CBP/p27KIP1 mice. As scheduled in our proposal, we have generated CBP/p27KIP1 compound knockout mice in the past funding year. Moreover, more than 120 additional mice, including CBP/p27KIP1 double knockout male mice and their control littermates, are currently maintained in our laboratory in order to monitor tumor formation and progression.

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Prostate cancer

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CBP and p27KIP1 in Prostate Carcinogenesis

1. Introduction

Prostate cancer is a very complex disease. Carcinogenesis of the prostate is hypothesized to be a multi-step process resulting from the progressive accumulation of genetic lesions. For example, expression of p27KIP1 is often decreased in human prostate cancer. Deletion of one or two alleles of p27KIP1 markedly enhances tumor formation and progression in the prostate of PTEN or Nkx3.1 knockout mice. However, deletion of p27KIP1 alone does not promote tumor development in the mouse prostate. Another example is CBP, which is also called cAMP response element binding protein (CREB)-binding protein. Studies from our laboratory and many others have shown that CBP is frequently lost or decreased in human primary prostate tumors, indicating that CBP is a prostate cancer-relevant gene. Indeed, our data demonstrate that prostate-specific knockout of CBP leads to the development of low-grade prostate carcinoma [prostate intraepithelial neoplasia (PIN) lesion] in aged mice. These findings suggest that additional genetic defect(s) are necessary for the development of high-grade tumors in prostates lacking CBP. Because both CBP and p27KIP1 are simultaneously lost or reduced in human prostate cancer, in this proposed three-year project we aim to determine whether loss of CBP works synergistically with decreased expression of $p27^{KIP1}$ in prostate carcinogenesis. We propose to introduce one or two p27KIP1-null alleles into mice with prostate-specific deletion of CBP. There are three Aims in this project: (1) Generate CBP knockout mice with concomitant deletion of p27KIP1 in the prostate; (2) Determine whether tumors in CBP/p27KIP1 double knockout mice progress at an accelerated rate compared to those in CBP single knockout mice; and (3) Determine whether inhibitors of histone deacetylases and proteasomes have therapeutic effects on tumors developed in CBP/p27KIP1 double knockout mice. In the past funding year, we have generated mice with the genotypes proposed in Aim 1.

2. Body

2.1. Mouse breeding

Table 1. Six groups of mice proposed to be generated in the proposal

Group #	Genotype	Experimental or Control
1	PB-$Cre4^{+}$/$CBP^{loxp/loxp}$/$p27KIP1^{+/+}$	Experimental
2	PB-$Cre4^{+}$/$CBP^{loxp/loxp}$/$p27KIP1^{+/-}$	Experimental
3	PB-$Cre4^{+}$/$CBP^{loxp/loxp}$/$p27KIP1^{-/-}$	Experimental
4	PB-$Cre4^{-}$/$CBP^{loxp/loxp}$/$p27KIP1^{+/+}$	Control
5	PB-$Cre4^{-}$/$CBP^{loxp/loxp}$/$p27KIP1^{+/-}$	Control
6	PB-$Cre4^{-}$/$CBP^{loxp/loxp}$/$p27KIP1^{-/-}$	Control

In order to generate six groups of mice with the desired genotypes as indicated in Table 1, PB-$Cre4^{+}$/$CBP^{loxp/+}$/$p27KIP1^{+/-}$ male mice and PB-$Cre4^{-}$/$CBP^{loxp/+}$/$p27KIP1^{+/-}$ female mice were generated by cross breeding PB-$Cre4^{+}$/$CBP^{loxp/loxp}$ male mice with $p27KIP1^{+/-}$ female mice, both of which are maintained in our laboratory. PB-$Cre4^{+}$/$CBP^{loxp/+}$/$p27KIP1^{+/-}$ male mice and PB-$Cre4^{-}$/$CBP^{loxp/+}$/$p27KIP1^{+/-}$ female mice were further cross bred to generate male mice PB-$Cre4^{+}$/$CBP^{loxp/loxp}$/$p27KIP1^{+/+}$; PB-$Cre4^{+}$/$CBP^{loxp/loxp}$/$p27KIP1^{+/-}$; PB-$Cre4^{+}$/$CBP^{loxp/loxp}$/$p27KIP1^{-/-}$ and their control male littermates PB-Cre4^{-}/CBP$^{loxp/loxp}$/p27KIP1$^{+/+}$; PB-Cre4^{-}/CBP$^{loxp/loxp}$/p27KIP1$^{+/-}$; PB-Cre4^{-} /CBP$^{loxp/loxp}$/p27KIP1$^{-/-}$. All these mice were then subjected to genotyping.

2.2. Genotyping

The genotypes of the mice were determined using a PCR-based approach. The schematic diagram in Figure 1 shows the CBP alleles of CBP mice as described previously (Kang-Decker et al., 2004).

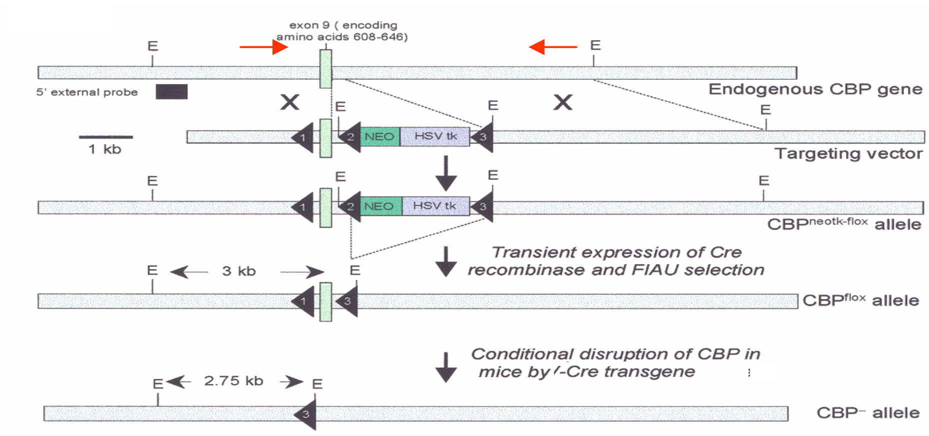

Figure 1. Schematic diagram of the CBP alleles. Trangles: loxp sites; arrows in red: primers for PCRs.

The expected sizes of the PCR amplicons for the loxp, wild-type or deleted CBP alleles are 1250 bp, 1100 bp, and 970 bp, respectively. Genomic DNA was isolated from mouse tails and subjected to PCR amplification. As demonstrated in Figure 2, PCR results from mice such as D202 and D203 show one upper band (1250 bp) and one lower band (1100 bp), indicating that those are CBP heterozygous mice. In contrast, PCR results from mice, such as D217 and D224 that are CBP loxp homozygous only show one upper band whereas wild-type mice such as D215 only have one lower PCR band. Thus, using the PCR-based approach, we have been very effective to identify mice with distinctive genotypes.

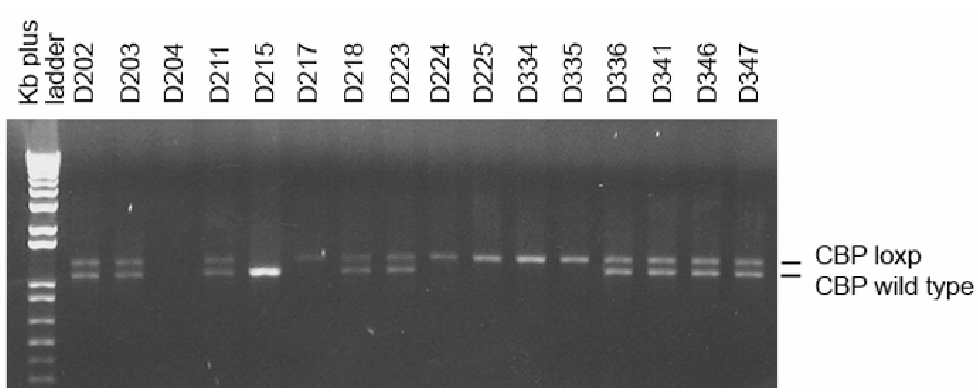

Figure 2. PCR-based genotyping of CBP wild-type, loxp heterozygous and homozygous mice.

The schematic diagram in Figure 3 shows the p27KIP1 alleles in p27KIP1 knockout mice as described previously (Fero et al., 1996). PCR amplification with the K5/K3 pair of primers generates an approximately 1500-bp PCR product from the wild-type p27KIP1 allele. PCR amplification with the N1/K3 pair of primers produces an unique 650-bp band from the knockout p27KIP1 allele.

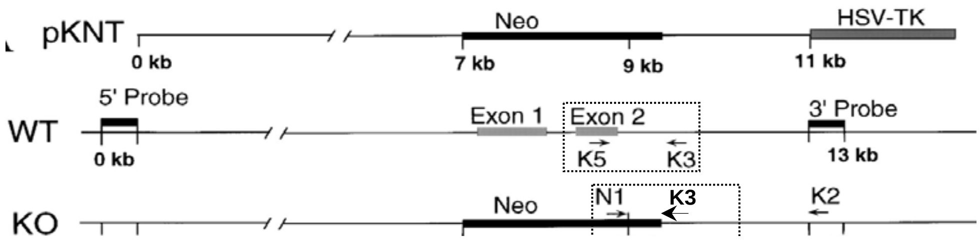

Figure 3. Schematic diagram of the wild-type and knockout alleles of p27KIP1. Arrows show the primers for wild-type and knockout PCRs. KO, knockout.

Genomic DNA was isolated from mouse tails. DNA samples from each mouse were used for PCR amplification with two pairs of primers (primers K5 and K3 for the wild-type allele, and N1 and K3 for the knockout allele). Figure 4 are the representative PCR results for p27KIP1 alleles. For example, D217 is a p27KIP1 wild-type mouse that is K5/K3 amplification positive but N1/K3 negative; D218 is p27KIP1 heterozygous and positive in amplifications with both K5/K3 and N1/K3 pairs of primers; D225 is a p27KIP1 homozygous knockout mouse that is positive in amplification with the N1/K3 pair of primers but negative with the K5/K3 pair of primers.

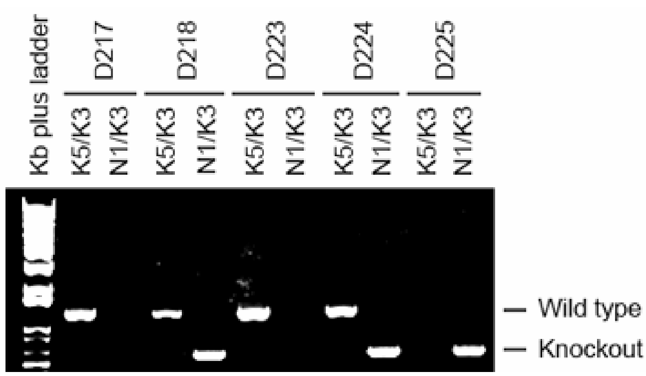

Figure 4. PCR-based genotyping of mice with wild-type and/or knockout alleles of p27KIP1.

2.3. Deletion of CBP and p27KIP1 genes in the prostate

In order to ensure the deletion of CBP and p27KIP1 genes in the mouse prostate, genomic DNA was isolated from different tissues of mice and subjected to PCR amplification. Figure 5 shows the PCR typing results from a CBP loxp heterozygous mouse (PB-Cre4^{+}/CBP$^{loxp/+}$/p27KIP1$^{-/-}$). Deletion of the CBP gene was evident by the amplification of the deleted CBP allele (970 bp) specifically in the prostate but not in other tissues examined. Due to the nature of germ-line deletion, loss of p27KIP1 was detected in all the tissues examined (data not shown). Thus, these results indicate that both CBP and p27KIP1 genes can be deleted in the prostate.

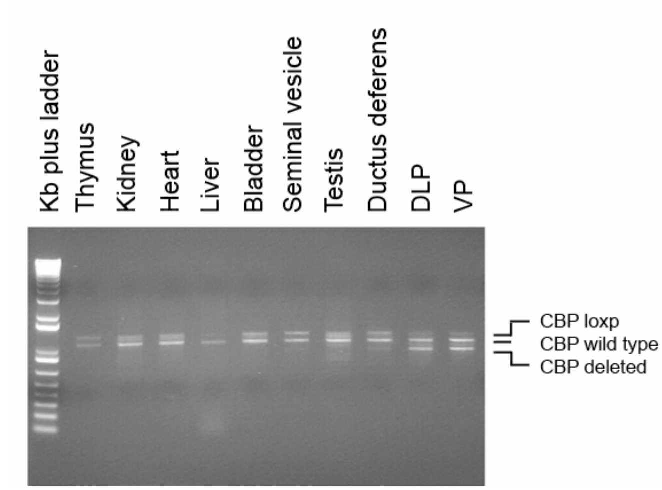

Figure 5. PCR results for CBP alleles in a PB-Cre4^{+}/CBP$^{loxp/loxp}$/p27KIP1$^{-/-}$ mouse. The sizes of the PCR products for loxp, wild-type and deleted CBP alleles are 1250 bp, 1100 bp, and 970 bp, respectively. DLP, dorsal and lateral prostate. VP, ventral prostate.

2.4. Deletion of CBP and p27KIP1 proteins in the prostate

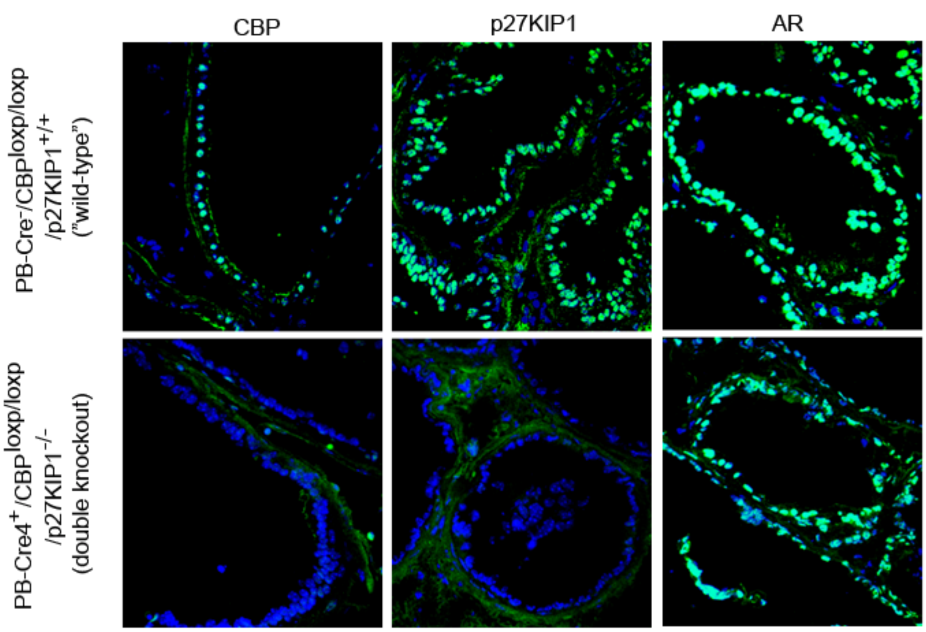

Figure 6. Immunofluorescence chemistry of CBP, p27KIP1 and androgen receptor (AR) in the dorsal and lateral prostates of "wild-type" (PB-Cre4^{-}/CBP$^{loxp/loxp}$/p27KIP1$^{+/+}$) and double knockout (PB-Cre4^{+}/CBP$^{loxp/loxp}$/p27KIP1$^{-/-}$) mice.

In order to determine the deletion of CBP and p27KIP1 proteins in the prostate, we performed immunofluorescence histochemistry with prostate tissues obtained from double knockout (PB-Cre4^{+}/CBP$^{loxp/loxp}$/p27KIP1$^{-/-}$) and "wild-type" control (PB-Cre4^{-}/CBP$^{loxp/loxp}$/p27KIP1$^{+/+}$) mice. Two-month-old mice were sacrificed. Prostate tissues were dissected out and snap frozen. Frozen tissue sections were stained with rabbit polyclonal antibodies for CBP (C-1, Santa Cruz) and p27KIP1 (C-19, Santa Cruz). As expected, nuclear staining of CBP protein was detected in the prostate of the "wild-type" mouse (Figure 6, upper left panel). However, little or no CBP proteins were detected in the prostate of the knockout mouse (Figure 6, lower left panel). Similar to the previous reports (Waltregny et al., 2001; Shim et al., 2003), p27KIP1 immunoreactivity in the normal prostatic epithelial cells was primarily nuclear with some staining in the cytoplasm (Figure 6, upper middle panel). However, no staining of p27KIP1 protein was detected in the prostate of the double knockout mouse (Figure 6, lower middle panel). As a control, the androgen receptor (AR) protein was detected in both "wild-type" and CBP/p27KIP1 knockout prostates (Figure 6, right panels). Thus, these data indicate that both CBP and p27KIP1 proteins are effectively deleted in the prostates of double knockout mice. These findings also strongly indicate that the PCR-based genotyping approach is efficient and accurate and that the radiation-based Southern blot hybridization method appears to be redundant and unnecessary.

3. Key Research Accomplishments

1. As planned in Aim 1, we have successfully generated mice with targeted deletion of CBP and p27KIP1 genes in the prostate as well as the control littermates.
2. Deletion of CBP and p27KIP1 genes in the prostates of knockout mice was ensured by using a PCR-based technique.
3. Deletion of CBP and p27KIP1 proteins in the prostates of knockout mice was further confirmed by immunofluorescence chemistry.
4. A cohort of CBP/p27KIP1 double knockout mice and their control littermates are currently maintained in our laboratory in order to determine whether compound deletion of CBP and p27KIP1 promotes tumor formation in the prostate in an accelerate rate than in CBP single knockout mice as proposed in Aim 2.

4. Reportable Outcomes

As summarized in Table 2, more than 120 additional mice with various genotypes have been generated in the past funding year. These mice will be maintained to monitor the tumor formation in the prostate in order to complete the second task of the proposal.

Table 2. CBP/p27KIP1 double knockout and control mice generated to date

Group #	Genotype	# of mice
1	PB-Cre4^{+}/CBP$^{loxp/loxp}$/p27KIP1$^{+/+}$	19
2	PB-Cre4^{+}/CBP$^{loxp/loxp}$/p27KIP1$^{+/-}$	22
3	PB-Cre4^{+}/CBP$^{loxp/loxp}$/p27KIP1$^{-/-}$	28
4	PB-Cre4^{-}/CBP$^{loxp/loxp}$/p27KIP1$^{+/+}$	18
5	PB-Cre4^{-}/CBP$^{loxp/loxp}$/p27KIP1$^{+/-}$	20
6	PB-Cre4^{-}/CBP$^{loxp/loxp}$/p27KIP1$^{-/-}$	23

5. Conclusion

By starting with PB-Cre4^{+}/CBP$^{loxp/loxp}$ and p27KIP1$^{+/-}$ as parental mice, we have generated CBP/p27KIP1 double knockout mice. Deletion of CBP and p27KIP1 genes and proteins in the mouse prostate was confirmed by PCR and immunofluorescence histochemistry. Moreover, more than 120 mice with various genotypes as proposed in

Task 1 have been generated. These mice will be maintained in order to monitor tumor formation. Thus, we have successfully completed the Task 1 proposed in Aim 1 in the past funding year. In the second funding year, we will characterize tumors in the prostates of mice as proposed in Task 2. Furthermore, we will continue crossbreeding experiments to generate additional 180 mice for Task 3 in order to determine whether inhibitors of histone deacetylases and proteasomes have therapeutic effects on tumors developed in CBP/p27KIP1 double knockout mice.

6. References

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7. Appendices

No.